Journal of Forestry Research (2013) 24(2): 325–332
DOI 10.1007/s11676-013-0356-7

ORIGINAL PAPER

Physiological responses of needles of *Pinus massoniana* elite families to phosphorus stress in acid soil

HE You-lan • LIU Ai-qin • Mulualem Tigabu • WU Peng-fei
MA Xiang-qing • WANG Chen • Per Christer Oden

Received: 2012-05-12; Accepted: 2012-06-13

Abstract: *Pinus massoniana* Lamb. is a major timber species widely planted in the South China, where the soil is acidic and deficient in phosphorus (P) due to fixation by aluminum and iron. Understanding the physiological responses to rhizospheric insoluble P is essential for enhancing plantation productivity. Thus, a sand culture experiment was conducted with four levels of P treatment (0, 5, 20 g insoluble P and 10 g soluble P), and 11 *P. massoniana* elite families. Physiological responses were measured after two months of stress. Compared to the normal soluble P treatment, the insoluble P treatment significantly reduced the proline content and the APase activity in the needles, while it significantly increased the catalase activity by 1.3-fold and malondialdehyde content by 1.2-fold. Soluble protein content was unaffected by the treatments, but chlorophyll content was significantly lower in P-deprived treatment compared with soluble and insoluble P treatments. These physiological responses also exhibited highly significant variation among families ($p < 0.01$). The findings suggest that increased catalase activities in the presence of insoluble P might be involved in the activation of an anti-oxidation defense mechanism that scavenges the reactive oxygen species elicited by the stress. And this response has a strong genetic control that can be exploited to identify desirable genotypes.

Keywords: Masson pine; phosphorus stress; anti-oxidative system; lipid peroxidation

Fund project: The study was founded by Nature Science Foundation of Fujian, China (Grant No. 2008J0038).
The online version is available at http:// www.springerlink.com

HE You-lan• LIU Ai-qin (✉)•WU Pengfei • MA Xiang-qing • WANG Chen
Forestry College, Fujian Agriculture and Forestry University, Fujian Province, Fuzhou 350002, P. R. China. Email: fjlaq@126.com

Mulualem Tigabu • Per Christer Oden
Swedish University of Agricultural Sciences, Faculty of Forest Science, Southern Swedish forest Research Centre, PO Box 49, SE-230 53 Alnarp, Sweden.

Corresponding editor: Zhu Hong

Introduction

Phosphorus (P) is an essential element for plant growth (Raghothama 1999), as it plays an important role in many physiological processes (Raghothama and Karthikeyan 2005). But P is often unavailable because it rapidly forms insoluble complexes with cations, particularly aluminum (Al) and iron (Fe) under acid conditions (Raghothama 1999; Raghothama and Karthikeyan 2005). P deficiency is, thus, considered to be one of the major limitations for plant growth and productivity, particularly in the tropics, where the soil is highly weathered with limited availability of P. Low P stress coupled with metal toxicity disrupts a number of physiological processes; for example through increased production of reactive oxygen species (ROS), which induce oxidative stress, leading to cell death in plants (Barceló and Poschenrieder 2002; Kochian et al. 2004).

Long-term adaptation to P-deficient environments enables some plants to evolve physiological mechanisms to cope with P deficiency (Kochian et al. 2004; Poschenrieder et al. 2008). Among others, a suite of antioxidant mechanisms is activated (Foyer and Noctor 2005; Giannakoula et al. 2011) to scavenge the elicited ROS (Veljovic-Jovanovic et al. 2006). For example, the increased activity of catalases in needles of hinoki cypress (*Chamaecyparis obtusa*) seedlings has been observed in response to Al toxicity (Ogawa et al. 2000). The increased accumulation of the amino acid proline (Pro) during rhizospheric Al stress was also observed and opined to act as a ROS scavenger (Sharma and Dietz 2006; Ashraf and Foolad 2007; Giannakoula et al. 2011). In addition, plants with tolerant to low P environment have developed mechanisms to enhance their P use efficiency by reducing tissue P requirements and efficient P remobilization from senescent or nonproductive tissues to growing or productive tissues (Vance et al. 2003; Kochian et al. 2004). Acid phosphatase (APase) secreted to the rhizosphere is believed to liberate P from organic sources in the soil (Gaume et al. 2001; Ko-

chian et al. 2004), and remobilize P from metabolically less active sites such as old leaves and vacuoles to young and developing tissues (Schachtman et al. 1998), as it is capable of hydrolyzing orthophosphate monoesters into more mobile orthophosphate anions (Vincent et al. 1992). In addition, *Pinus massoniana* Lamb. is known to form symbiotic association with several mycorrhizae (Shi et al. 2006) that play key roles in P absorption and translocation pathways (Gao et al. 2009; Wang and Ding 2011). Despite increasing evidence for root morpho-physiological responses to rhizospheric P-stress, it is not known if similar physiological adaptive responses are triggered in the shoots, and if these responses are genetically controlled.

Thus, this study was conducted to examine physiological changes in needles of 11 elite families of *P. massoniana*, a major fast growing pioneer tree species in South China, where the soil is acidic and deficient in available P due to fixation by aluminum and iron (Chen et al. 1996). Unlike previous studies that mainly used KH_2PO_4 as P sources under the water culture and pot experiments (Xie and Zhou 2002; Xie et al. 2005; Zhou et al. 2003, 2004, 2005; Xu and Ding 2006; Yu et al. 2007), we conducted a sand culture experiment using insoluble P (Al-P and Fe-P) similar to the natural condition in acid soils of southern China. The experiment involved four P treatments: No insoluble P (0 g $AlPO_4$ and $FePO_4$), low insoluble P (5 g, 1:1 $AlPO_4$ and $FePO_4$), high insoluble P (20 g, 1:1 $AlPO_4$ and $FePO_4$), and normal P supply (KH_2PO_4), and 11 elite *P. massoniana* families from different geographic regions of China. After two months of stress experiment, proline content, malondialdehyde content, catalase activity, APase activity (APase), soluble protein and chlorophyll contents of needles were determined and analyzed. The hypotheses of the study were (1) low P stress induces membrane lipid oxidation, but the activity of antioxidants might increase to scavenge the reactive oxygen species in families that are adapted to low P conditions; (2) the activity of APase in needles will be higher in families adapted to low P stress than in sensitive families because it remobilizes P from senesce tissues; and (3) low P stress reduces the content of soluble proteins and total chlorophyll compared to normal P supply, but the reduction varies by family.

Materials and methods

Plant materials

Seeds of 11 elite *P. massoniana* families (No.2, 242, 335, 388, 568, 658, 659, 587, 474, 326, and local seed source as a control) were collected separately from a State-owned seed orchard in Zhangping, Fujian Province, P. R. China, which was established using materials from different geographic regions. Seedlings from each family were raised in containers individually for six months in the glasshouse at the College of Forestry, Fujian Agriculture and Forestry University. The environmental conditions in the glasshouse were as follows: 29.3 °C/23 °C (day/night); photon flux density of 21 $mol \cdot m^{-2} \cdot d^{-1}$, and 42.7% and 67.7% relative humidity during the light and dark period of the experiment, respectively.

Seedlings of similar size (10 cm of shoot height) were selected as experimental materials and transplanted, after washing the roots with water, in polyethylene pots (15 cm of diameter, 18 cm at height) filled with a mixture of vermiculites, peat soils and sands (3 kg per pot) at 1:1:1 volume ratio. The pH value, total N, total P and total K of the substrate was 4.56, 1.53 $g \cdot kg^{-1}$, 1.53 $g \cdot kg^{-1}$ and 21.48 $g \cdot kg^{-1}$, respectively. The seedlings were left to grow under normal condition for a month in order to reduce internal phosphorus concentrations to appropriate lower levels. Thereafter the phosphorus stress treatments were invoked.

Experimental design

To examine changes in needle physiology of different *P. massoniana* families in response to insoluble P source in acid soil, the following treatments were applied: No insoluble P (P_0: 0 g, $AlPO_4$ and $FePO_4$), low insoluble P (P_5: 5 g, $AlPO_4$ and $FePO_4$), high insoluble P (P_{20}: 20 g, $AlPO_4$ and $FePO_4$), and normal P supply (CK: 10 g KH_2PO_4). The insoluble P was applied as $AlPO_4$ and $FePO_4 \cdot 4H_2O$ (1:1). The different P treatments were fully mixed with sand and added into the growing medium. The seedlings (one seedling per pot) were then left to grow under treatment conditions for two months, and the experiment was replicated three times.

To meet the needs for other nutrients for growth of *P. massoniana* seedlings, macro-nutrients were added to each pot according to a modified Hoagland solution (Mao 2005). This solution contained 0.51 $g \cdot L^{-1}$ of KNO_3, 0.82 $g \cdot L^{-1}$ $Ca(NO_3)_2$, 0.49 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$, 0.136 $g \cdot L^{-1}$ KH_2PO_4. Micro-nutrients were also supplied according to Arnon formula: 2.86 $g \cdot L^{-1}$ H_3BO_3, 0.08 $g \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.22 $g \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 1.81 $g \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.09 $g \cdot L^{-1}$ $H_2MoO_4 \cdot H_2O$ and 20 $g \cdot L^{-1}$ Fe_2EDTA. KCl was used as a substitute for KH_2PO_4 in insoluble phosphate treatments at the same amount. The pH of the nutrient solution was adjusted to 6.0 (Mao 2005). The nutrient solutions (100 mL) were added into each pot every three days during the treatment period.

Measurements of physiological variables

After two months of stress, the needles were sampled for measuring the following physiological variables: proline content, malondialdehyde content, catalase activity, acid phosphatase activity, soluble protein content and photosynthetic pigment molecules. Proline (Pro) content was determined using the coloration method of acidic Ninhydrin hydrate (Ao et al. 2007). For each treatment, a sample of needles (0.25 g) per replication was taken, weighed and then ground in liquid nitrogen. To the ground samples we added 5 mL of 13% sulfosalicylic acid, then the homogenate was poured into centrifuge tubes, and extracted in boiling water bath for 10 min. After cooling in a test tube, 2 mL of the supernatant was taken, to which was added 2 mL glacial acetic acid and 3 mL of 2.5% ninhydrin. The mixture was then put in boiling water bath for 40 min for coloring. After cooling the mixture, 5 mL-toluene was added to extract red substances.

Take out the toluene phase, and then the absorbance value was measured at 520 nm wavelength. Pro content ($\mu g \cdot g^{-1}$, FW) was determined by comparison with standard curves.

Malondialdehyde (MDA) content was also determined with a coloration method using thiobarbituric acid (Ao et al. 2007). For each treatment, a sample of needles (0.25 g) per replication was taken, weighed and then ground in liquid nitrogen. To the ground samples were added 4 mL of 5% thiobarbituric acid (TCA). The homogenate was transferred into centrifuge tubes and centrifuged for 10 min at 4500 $r \cdot min^{-1}$. We took l.5 mL-supernatant and added 2.5 mL of 0.5% TBA, and the mixture was put in boiling water bath for 10 min. Thereafter, the mixture was removed and the reaction terminated in ice bath (about 5 min), and then centrifuged again for 10 min at 4500 $r \cdot min^{-1}$. Finally the supernatant was taken and the absorbance value was measured at 532 nm and 600 nm, with distilled water as control.

Catalase (CAT) activity was measured using an ultraviolet absorption method (Li et al. 2007). A known quantity of needles (0.25 g) was ground in liquid nitrogen, to which was added 4 mL pre-cooling phosphate buffer with pH of 7.0. The homogenate was transferred into centrifuge tubes and centrifuged for 15 min at 4000 $r \cdot min^{-1}$. The enzyme extract (0.3 mL) was taken from the centrifuge tube and 1.5 mL of Tris-HCl reagent with pH of 7.0 and 1 mL distilled water was added. The mixture was preheated for 3 min at 25 °C (adjusted at room temperature) water bath and then 0.2 mL of 0.2 $mol \cdot L^{-1}$ H_2O_2 was added into each tube, shaken rapidly and poured into a colorimetric dish and absorbance at 240 nm was measured after 30 s from the onset of recording, and once thereafter every 1 min for 3 min. Catalase activity was determined using enzyme activity per unit that A_{240} reduced 0.1 amount of enzyme within 1 min (U).

Acid phosphatase (APase) activity was determined following the McLachlan (1980) method with slight modifications. Needle samples (0.25 g each from each treatment) were ground separately in liquid nitrogen, and then 4 mL pre-cooling extract of 0.1 $mol \cdot L^{-1}$ NaAc with pH of 5.2 was added, extracted for 1 h in ice bath, and then centrifuged for 25 min at 7200 $r \cdot min^{-1}$. APase reaction system consisted of 0.9 ml of 0.1 $mol \cdot L^{-1}$ NaAc with pH of 5.2, 0.9 mL of 1 $mmol \cdot L^{-1}$-Nitro phenol phosphate (pNPP) and 0.9 mL of 5 $mmol \cdot L^{-1}$ $CaCl_2$. Then we took 0.3 mL of APase enzyme solution. A total of 3 mL-reaction mixture was heated at 37 °C water bath for 30 min. The reaction mixture was removed from the water bath and 1 mL of 1 $mol \cdot L^{-1}$ NaOH was immediately added to terminate enzymatic reaction. The mixture was then centrifuged for 25 min at 7200 $r \cdot min^{-1}$ at 4 °C, thereafter absorbance was measured at 405 nm. APase activity ($\mu g \cdot g^{-1} \cdot min^{-1}$) was assessed by the amount of NPP generated from the needles fresh weight hydrolyzed pNPP in unit time.

Soluble protein content was determined using the Coomassie Brilliant Blue method (Zheng 2006). A known amount of needles (0.25 g) were ground in liquid nitrogen and 4 mL pre-cooled distilled water was added, afterwards the homogenate was transferred into centrifuge tubes and centrifuged for 15 min at 4000 $r \cdot min^{-1}$. The supernatant (1 mL) was taken and 5 mL of G-250 reagent of Coomassie Brilliant Blue was added. After shaking well and letting settle for 2 min, the absorbance was measured at 595 nm using distilled water as blank. Protein content ($mg \cdot g^{-1}$, FW) of samples was determined by comparing with standard protein curves.

The total chlorophyll content was determined by the ethanol-acetone extraction method (Zhang 1986). We took 0.05 g pine needles from each treatment, cut them into small pieces (2–3 mm, long), placed the pieces in a tube and added 10 mL of ethanol-acetone mixture (1:1 v/v). The tubes were placed in the dark under room temperature (25 °C) until the materials turned completely white (shaken several times during the period). Photosynthetic pigment contents was expressed in $mg \cdot g^{-1}$, FW.

Data analysis

Prior to the analysis of variance (ANOVA), data sets that violated the homoscedasticity assumption were log-transformed. Thereafter, Two-Way ANOVA was performed to examine differences in physiological parameters among *P. massoniana* families and phosphorus stress treatments using the following general linear model:

$$Y_{ijk} = \mu + F_i + T_j + F_i T_j + e_{ijk} \quad (1)$$

where, Y_{ijk} is the response variable, μ is the overall mean, F_i is the effect of families, T_j is the effect of phosphorus stress treatments, $F_i T_j$ is the treatment by family interaction, and e_{ijk} is the error term with k replicates. The parameters T_i, F_j and $T_i F_j$ were treated as fixed effects. When significant interaction effect was observed, One-Way ANOVA for each family was performed to detect differences among phosphorous stress treatments separately. Means that exhibited significant differences were compared using Tukey's honestly significant test at 5% level. All statistical analyses were done using the SPSS 17 software package (SPSS 17 for Windows, Release 2009 Chicago: SPSS Inc.).

Results

Anti-oxidant system

The Pro content, MDA level and CAT activity varied significantly between *P. massoniana* families ($p < 0.01$), P treatments ($p < 0.01$) and their interaction ($p < 0.01$). Comparison of main effects of P treatments and families revealed that the mean Pro content was lower in the presence of insoluble P (11.02 $\mu g \cdot g^{-1}$, FW in P_{20} and 13.03 $\mu g \cdot g^{-1}$, FW in P_5) than in P-deprived (16.54 $\mu g \cdot g^{-1}$, FW) and normal P supply conditions (17.22 $\mu g \cdot g^{-1}$, FW), and the highest Pro content was recorded for families No. 335 (26.52 $\mu g \cdot g^{-1}$, FW) and 474 (24.38 $\mu g \cdot g^{-1}$, FW) and the lowest for families No. 659 (8.15 $\mu g \cdot g^{-1}$, FW) and 658 (8.53 $\mu g \cdot g^{-1}$, FW). Further analysis of the Pro content of each family across different P treatments using One-Way ANOVA revealed that five families (No. 388, 568, local, 242 and 658) were insensitive to the P treatments; while the other families had significantly less Pro content in the presence of insoluble P compared to the normal P supply, particularly families 2 and 659 had substantially lower Pro content (Fig. 1).

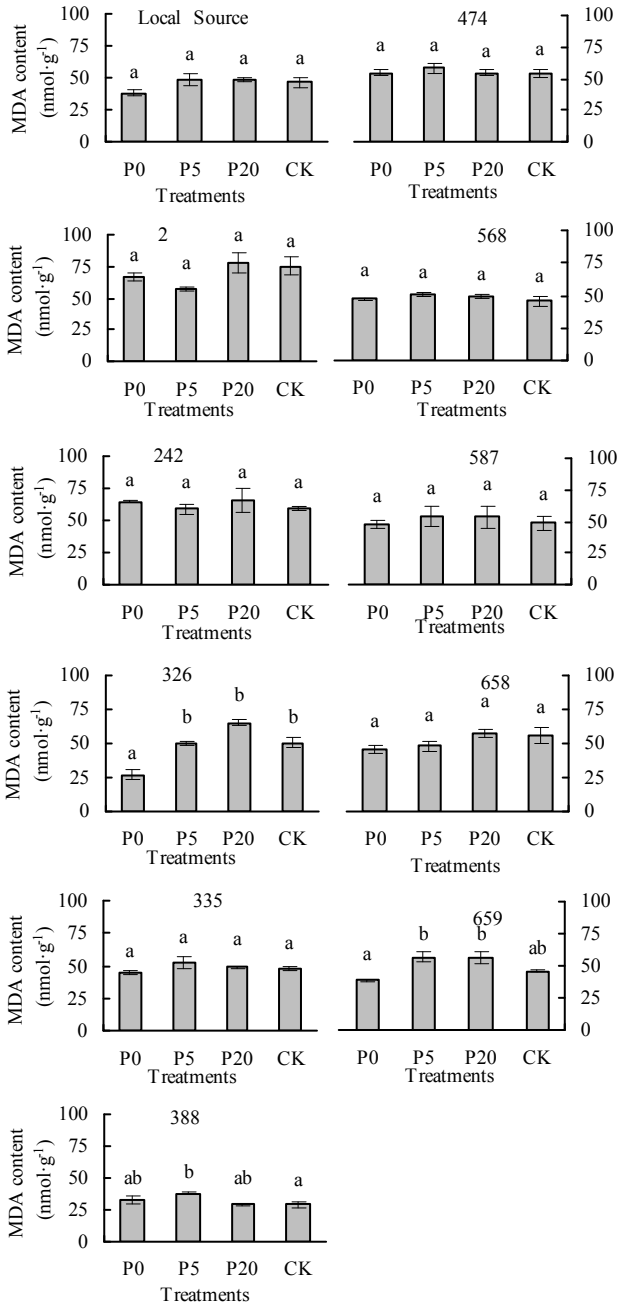

Fig. 1 Proline content (μg·g^{-1}FW) in needles of different *Pinus massoniana* families supplied with no insoluble P (P_0), low level of insoluble P (P_5), high level of insoluble P (P_{20}) and normal level of P (CK). For each family, bars (means ± SE) followed by the same lower case letter (s) are not significantly different at 5% level.

Comparison of main effects of P treatments and families showed that the mean MDA content was significantly higher in seedlings exposed to both high (51.19 nmol·g^{-1}) and low (48.62 nmol·g^{-1}) levels of insoluble P treatments than P-deprived treatment (43.98 nmol·g^{-1}), whereas the normal P supply did not bring significant effect on MDA content (47.18 nmol·g^{-1}) compared to the insoluble P treatments, although it resulted in significantly higher MDA content compared to the P-deprived treatment. The MDA content of *P. massoniana* was the highest for family No. 2 (64.52 nmol·g^{-1}) and the lowest for family No. 388 (30.42 nmol·g^{-1}). Further analysis of the MDA content of each family across different P treatments using One-Way ANOVA showed that only three families (No. 326, 388 and 659) exhibited significant differences in MDA content (Fig. 2).

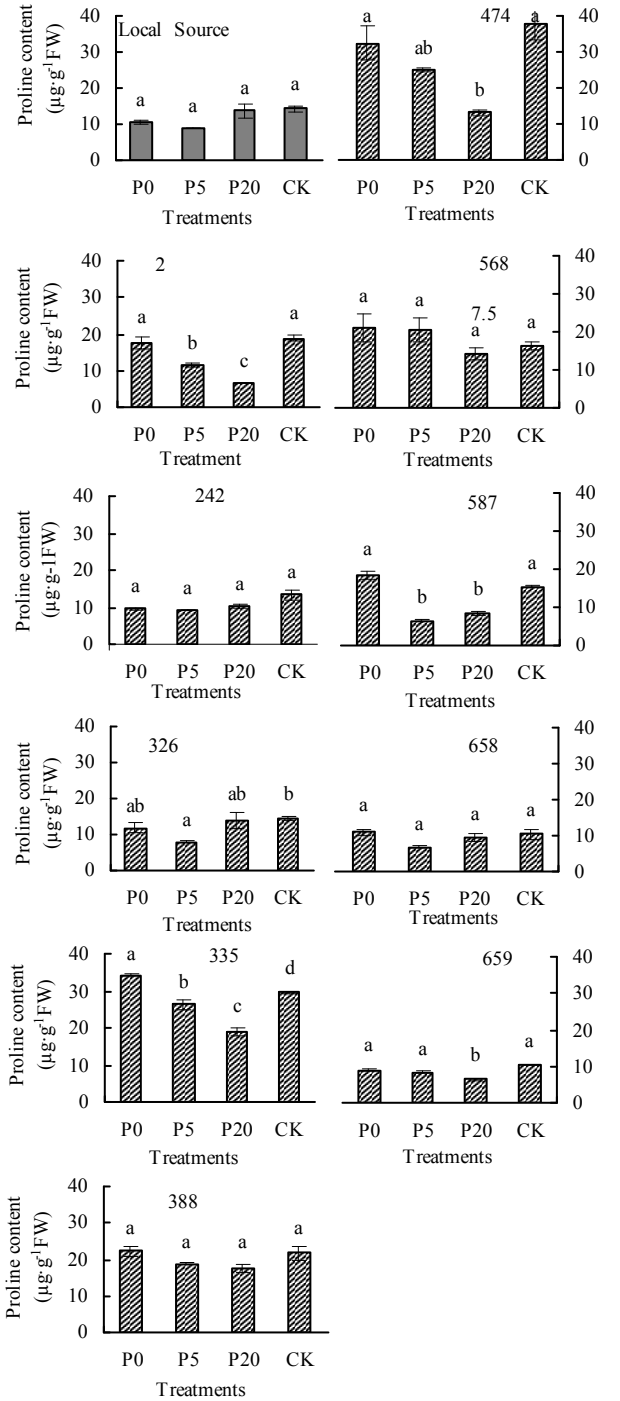

Fig. 2 MDA content (nmol·g^{-1}) in needles of different *Pinus massoniana* families supplied with no insoluble P (P_0), low level of insoluble P (P_5), high level of insoluble P (P_{20}) and normal level of P (CK). For each family, bars (means ± SE) followed by the same lower case letter (s) are not significantly different at 5% level.

For family No. 659, the insoluble P treatments resulted in significantly higher MDA content than the P-deprived treatment, while the normal P supply did not bring comparative advantage over the P stress treatments. For family No. 326, the MDA content was significantly higher in all P treatment except the P-deprived treatment that resulted in significantly lower MDA content. For family No. 388, low level of insoluble P supply resulted in significantly higher MDA content compared to the normal P supply.

Comparison of main effects of P treatments and families showed that the mean CAT activity was significantly higher for seedlings exposed to a high level of insoluble P (243.56 $U \cdot g^{-1} \cdot min^{-1}$) than seedlings grown under P-deprived condition (178.84 $U \cdot g^{-1} \cdot min^{-1}$), normal P supply (194.20 $U \cdot g^{-1} \cdot min^{-1}$) and low level of insoluble P supply (205.17 $U \cdot g^{-1} \cdot min^{-1}$). The CAT activity was the highest for family No. 335 (309.01 $U \cdot g^{-1} \cdot min^{-1}$) and the lowest for families No. 659 (13.21 $U \cdot g^{-1} \cdot min^{-1}$) and 474 (136.69 $U \cdot g^{-1} \cdot min^{-1}$). Examining the CAT activity of each family in response to different P treatments using One-Way ANOVA revealed similar responses in four families (No. 335, 242, 568, 587), significantly higher CAT activity in the presence of insoluble P than under normal P supply for four families (No. 658, local, 326, 2), and significantly lower CAT activity in P stress treatments than in the normal P supply treatment for families No. 388 and 659; although seedlings of family No. 659 had similar CAT activity in both normal P and high level of insoluble P supply treatments (Fig. 3).

Acid phosphatase activity

APase activity in needles varied significantly between *P. massoniana* families ($p < 0.01$) and P treatments ($p = 0.015$). Comparison of main effects of P treatments and families revealed that the mean APase activity was higher when seedlings were grown under normal P supply than under P-deprived condition, whereas seedlings under both high and low insoluble P supply did not show significant difference in APase activity, compared to the normal P supply and P-deprived conditions (Table 1). Mean APase activity was lowest for family No. 568. APase activity was highest for families No. 587, 474, 326 and 659. P treatments also showed significant interaction effects on APase activity in needles ($p < 0.01$). Further analysis of APase activities of each family across different P treatments using One-Way ANOVA showed that seven families (No. 587, 474, 659, 335, 2, 658 and 568) had comparably similar APase activity across all P treatments (Table 1). The local family had higher APase activity in the presence of low level of insoluble P than other P treatments, while the opposite pattern was observed in family 388 (Table 1). Families No. 326 and 242 exhibited higher APase activity under normal P supply than under P-deprived condition; and lower APase activity in needles of family 326 was also observed under high level of insoluble P supply, compared to the normal P supply (Table 1).

Soluble protein

Soluble protein content of needles varied significantly between families ($p < 0.01$). The mean protein content, pooled over all levels of P treatment, was higher for families No. 335, 474, 587 and 242, compared to families No. 658, 659 and the local family (Table 2).

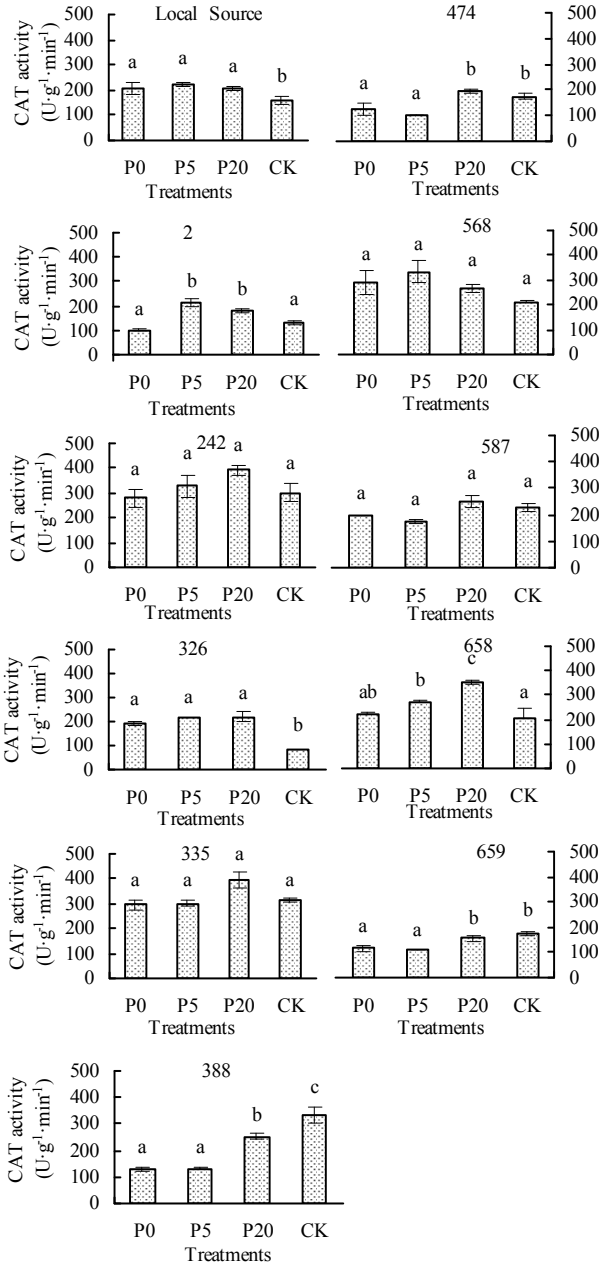

Fig. 3 CAT activity ($U \cdot g^{-1} \cdot min^{-1}$) in needles of different *Pinus massoniana* families supplied with no insoluble P (P_0), low level of insoluble P (P_5), high level of insoluble P (P_{20}) and normal level of P (CK). For each family, bars (means ± SE) followed by the same lower case letter (s) are not significantly different at 5% level.

Chlorophyll content

Chlorophyll content varied significantly between families, P treatments and their interaction ($p < 0.001$ for each factor and interaction effect). Comparison of main effects of P treatments and families revealed that the mean chlorophyll content was significantly lower for seedlings under P-deprived medium than

for seedlings either in normal P supply or under insoluble P supply (Table 3). Families with the lowest chlorophyll content were No. 659, 335 and 388, while those with the highest value were No. 474, 658, 326 and 2 (Table 3). When the chlorophyll content of each family was examined across the different P treatments separately, five families (No. 242, 474, 658, 659 and the local family) were found to be insensitive; two families (No. 326 and 335) had higher chlorophyll content under normal P supply and one family (No. 587) had lower chlorophyll content under normal P supply than P stress condition; while families No. 2 and 568 had higher chlorophyll content under low and high levels of insoluble P supply than under P-deprived condition (Table 3).

Table 1. APase activity (μg·g^{-1}·min^{-1}) in needles of different *Pinus massoniana* families supplied with no insoluble P (P_0), low level of insoluble P (P_5), high level of insoluble P (P_{20}) and normal level of P (CK)

Families	CK	P_0	P_5	P_{20}	Mean (F)
Local	115.66 ± 13.32 **a**	74.39 ± 5.47 **a**	191.35 ± 26.72 **b**	106.32 ± 9.32 **a**	121.90 ± 14.6 **A**
2	164.21 ± 5.27 **a**	112.16 ± 12.39 **a**	129.71 ± 6.08 **a**	143.39 ± 28.06 **a**	137.37 ± 8.9 **ABC**
242	177.78 ± 17.53 **b**	88.30 ± 8.08 **a**	115.44 ± 7.90 **ab**	135.21 ± 22.58 **ab**	129.18 ± 11.8 **AB**
326	184.56 ± 17.52 **b**	117.19 ± 7.71 **a**	165.26 ± 16.604 **ab**	121.75 ± 9.07 **a**	147.19 ± 10.4 **BC**
335	138.95 ± 4.71 **a**	125.73 ± 6.79 **a**	146.32 ± 29.98 **a**	147.37 ± 11.96 **a**	139.59 ± 7.6 **ABC**
388	168.07 ± 2.84 **b**	156.73 ± 10.77 **b**	102.46 ± 2.84 **a**	145.73 ± 13.07 **b**	143.25 ± 8.4 **ABC**
474	142.46 ± 24.39 **a**	192.28 ± 12.21 **a**	131.23 ± 6.04 **a**	151.35 ± 14.04 **a**	154.33 ± 9.6 **BC**
568	109.00 ± 7.42 **a**	117.19 ± 3.04 **a**	125.26 ± 9.93 **a**	107.37 ± 16.11 **a**	114.71 ± 4.9 **A**
587	171.70 ± 6.18 **a**	198.13 ± 24.45 **a**	133.33 ± 5.65 **a**	176.14 ± 28.66 **a**	169.83 ± 11.3 **C**
658	103.86 ± 6.18 **a**	115.21 ± 11.18 **a**	134.50 ± 18.14 **a**	158.01 ± 15.09 **a**	127.89 ± 8.4 **AB**
659	132.63 ± 1.27 **a**	132.63 ± 8.10 **a**	161.69 ± 16.81 **a**	159.30 ± 9.12 **a**	146.58 ± 6.1 **BC**
Mean (T)	146.25 ± 5.8 **A**	129.99 ± 7.1 **B**	139.69 ± 5.8 **AB**	141.08 ± 5.7 **AB**	

Notes: Means (± SE) showed by the same lower case letter (s) across the row, and overall means for families (F) and treatments (T) followed by the same upper case letter (s) are not significantly different at 5% level.

Table 2. Soluble protein contents (mg·g^{-1}FW) in needles of *Pinus massoniana* families supplied with no insoluble P (P_0), low level of insoluble P (P_5), high level of insoluble P (P_{20}) and normal level of P (CK)

Families	CK	P_0	P_5	P_{20}	Mean (F)
Local	2.21 ± 0.2	2.20 ± 0.1	2.51 ± 0.1	2.10 ± 0.1	2.26 ± 0.1 **A**
2	2.63 ± 0.1	2.41 ± 0.1	2.42 ± 0.3	2.38 ± 0.2	2.41 ± 0.1 **ABC**
242	2.57 ± 0.1	2.50 ± 0.1	2.66 ± 0.1	2.49 ± 0.2	2.56 ± 0.1 **BC**
326	2.19 ± 0.1	2.27 ± 0.2	2.44 ± 0.1	2.57 ± 0.3	2.37 ± 0.1 **ABC**
335	2.87 ± 0.02	2.71 ± 0.1	2.98 ± 0.1	2.79 ± 0.1	2.84 ± 0.1 **D**
388	2.24 ± 0.2	2.27 ± 0.03	2.32 ± 0.2	2.51 ± 0.04	2.34 ± 0.1 **AB**
474	2.74 ± 0.2	2.61 ± 0.1	2.66 ± 0.1	2.48 ± 0.01	2.62 ± 0.1 **CD**
568	2.35 ± 0.2	2.52 ± 0.1	2.61 ± 0.2	2.24 ± 0.1	2.43 ± 0.1 **ABC**
587	2.70 ± 0.1	2.47 ± 0.1	2.55 ±0.2	2.70 ± 0.01	2.60 ± 0.1 **CD**
658	2.43 ± 0.1	2.21 ± 0.1	2.13 ± 0.2	2.22 ± 0.2	2.25 ± 0.1 **A**
659	2.46 ± 0.03	2.19 ± 0.1	2.28 ± 0.2	2.11 ± 0.1	2.26 ± 0.1 **A**
Mean (T)	2.49 ± 0.1 **A**	2.39±0.04 **A**	2.49 ± 0.1 **A**	2.42 ± 0.1 **A**	

Grand means (± SE) followed by the same letter across the row for treatments and the column for families are not significantly different

Table 3. Chlorophyll content (mg·g^{-1}FW) of different *Pinus massoniana* families supplied with no insoluble P (P_0), low level of insoluble P (P_5), high level of insoluble P (P_{20}) and normal level of P (CK)

Families	CK	P_0	P_5	P_{20}	Mean (F)
Local	0.87 ± 0.05 **a**	0.95 ± 0.04 **a**	0.90 ± 0.08 **a**	0.95 ± 0.05 **a**	0.92 ± 0.03 **ABC**
2	1.15 ± 0.03 **ab**	0.95 ± 0.05 **a**	1.27 ± 0.09 **b**	0.93 ± 0.08 **a**	1.07 ± 0.05 **CDE**
242	1.08 ± 0.09 **a**	1.11 ± 0.02 **a**	0.87 ± 0.09 **a**	1.06 ± 0.10 **a**	1.03 ± 0.05 **BCDE**
326	1.33 ± 0.09 **b**	0.90 ± 0.08 **a**	0.96 ± 0.09 **a**	1.11 ± 0.02 **ab**	1.08 ± 0.06 **CDE**
335	1.07 ± 0.10 **b**	0.60 ± 0.01 **a**	0.71 ± 0.03 **a**	1.03 ± 0.07 **b**	0.85 ± 0.07 **AB**
388	1.09 ± 0.13 **b**	0.73 ± 0.03 **a**	0.85 ± 0.01 **ab**	0.78 ± 0.05 **a**	0.86 ± 0.05 **AB**
474	1.10 ± 0.20 **a**	1.06 ± 0.06 **a**	1.07 ± 0.03 **a**	1.39 ± 0.13 **a**	1.15 ± 0.07 **E**
568	1.25 ± 0.15 **b**	0.61 ±0.01 **a**	0.91 ± 0.11 **ab**	1.33 ± 0.10 **b**	1.03 ± 0.10 **BCDE**
587	0.69 ± 0.01 **a**	0.95 ± 0.12 **ab**	1.19 ± 0.06 **b**	0.99 ± 0.05 **b**	0.96 ± 0.06 **ABCD**
658	1.29 ± 0.13 **a**	1.02 ± 0.001 **a**	1.09 ± 0.05 **a**	1.04 ± 0.14 **a**	1.11 ± 0.05 **DE**
659	0.76 ± 0.07 **a**	0.71 ± 0.03 **a**	0.91 ± 0.04 **a**	0.88 ± 0.06 **a**	0.82 ± 0.03 **A**
Mean (T)	1.06 ± 0.04 **A**	0.87 ± 0.03 **B**	0.97 ± 0.03 **A**	1.04 ± 0.04 **A**	

Notes: Means (± SE) showed by the same lower case letter (s) across the row, and overall means for families (F) and treatments (T) followed by the same upper case letter (s) are not significantly different at 5% level.

Discussion

There is strong evidence in this study that rhizospheric low P stress induces a number of physiological changes in the needles with marked genetic variability, as it has been observed in the roots of several plants. The Pro content in the needles of *P. massoniana* was 1.6-fold higher under normal P supply than in the presence of high level of insoluble P (P_{20}) in the growing medium. One possible explanation would be that seedlings grown under normal P supply produce larger root biomass and have higher rate of water uptake to compensate water loss due to high rate of transpiration. Thus, the build-up of Pro in the needles could serve as a mechanism to regulate water along the soil-plant-atmosphere continuum.

In support of this argument, Singh et al. (2000) reported that white clover grown under high-P supply dries the soil to greater soil water suctions; thus their leaves have lower water potential values with fewer water stress symptoms, have greater osmotic adjustment, proline concentration and leaf expansion rates compared with clovers grown under low P supply. In the case of *P. massoniana,* mycorrhizal formation might play a role in regulating the uptake of water under low P stress conditions, as mycorrhizal association is one of the mechanisms by which plants cope with nutrient deficiency. Above half of the families investigated in this study produced higher levels of Pro in the needles under normal P supply than under insoluble P supply while the rest of the families had a similar level of Pro across the different P treatments. It is suggested that needle Pro may not be a good diagnostic tool for rhizospheric low P stress, as opposed to root proline. Pro is considered as an osmo-protectant, resulting in increased tolerance to osmotic and environmental stresses (Yoshiba et al. 1997).

In the present study, the addition of 5 g (P_5) or 20 g (P_{20}) $AlPO_4$ and $FePO_4$ into the growing media resulted in increased concentration of MDA by 1.11 and 1.16-fold, compared to P-deprived growing medium (P_0), indicating that the insoluble P supply coupled possibly with Al toxicity induced membrane lipid peroxidation in the needles as observed in roots of other plants (Basu et al. 2001; Giannakoula et al. 2011). Concomitantly, the CAT activity increased by 1.15 and 1.36-fold in P_5 and P_{20}, respectively, compared to P_0. This indicates that the low P stress environment not only enhances the production of reactive oxygen species (ROS) that attack the membrane lipids but also activates antioxidant enzymes that scavenge the produced ROS in the needles. The increased activity of CAT in response to rhizospheric stress has been observed in hinoki cypress needles (Ogawa et al. 2000) and tomato leaves (Wang et al. 2005). Similarly, increased MDA content and CAT activities in response to low P stress have been observed in pine needles (Xu and Ding 2006).

APase in leaf tissues is, thus, believed to be related to P use efficiency under P-limiting conditions. However, it has been demonstrated that the increased APase in leaves is often associated with the severity of P deficiency symptoms in the plant (McLachlan 1980); thus, it is argued that the increased leaf APase is merely an indication of P deficiency. In the present study, seven out of 11 families exhibited a similar level of APase activity under normal P supply and P-stress conditions, suggesting that needle APase may not play a major adaptive role to low P availability in this species. In support of this argument, Yan et al. (2001) reported the lack of a relationship between APase in leaves of common bean and P status of young, intermediate and old leaves, and argued that APase is not related to net P remobilization.

The local family, however, is exceptional to this conclusion, as the needle APase was significantly higher under insoluble P supply condition than under normal P supply. Since this family is adapted to acid soil in south China, enhanced APase might be a mechanism to remobilize tissue P from senescent or non-productive tissues to growing or productive tissues, as observed in other plants (Smith et al. 1990; Snapp and Lynch 1996).

There is no evidence in the present study that rhizospheric insoluble P affects the synthesis of soluble protein. The families might bypass P requiring steps during protein synthesis to optimize P efficiency, although we have not examined this aspect. However, the observed variation in soluble protein content of needles among families could be related to their protein synthesis efficiency. Rhizospheric insoluble P also had no impact on chlorophyll content, but rather total P-deprivation significantly reduced chlorophyll content, suggesting that the families might have a mechanism to activate fixed P to meet their P requirement. Our result is consistent with previous study that showed a decline in chlorophyll content under low P stress (Xu and Ding 2006).

Conclusions

It can be concluded that changes in MDA and CAT activities can serve as rapid diagnostic tools to rhizospheric insoluble P stress in *P. massoniana*. The increased catalase activities in the presence of insoluble P showed that the activation of anti-oxidant defense mechanisms scavenges the reactive oxygen species elicited by low P stress. This change, however, has shown a strong genetic variation that can be exploited to identify P efficient genotypes. In light of our findings, families No. 335 and 242 have the highest catalase activity in response to P-deficiency. Membrane lipid oxidation is the lowest in family No. 388 across all P treatments, while family No. 2 appears to be succumbing to membrane lipid oxidation as a result of P-deficiency, as shown by high MDA level and low catalase activities. Insoluble P does not trigger the accumulation of proline content and APase activities in needles, suggesting that these variables have less adaptive role to P stress in acid soils. This study provides a basis for further evaluation of the families in the field to select superior families for future plantation establishment.

Acknowledgements

We thank Xiaolong Hou for assistance with field sampling and Associate Professor Liping Cai for chemical analyses in the laboratory.

References

Ao JJ, Kang ZL, Yu Y. 2007. *Plant physiology experimental techniques*. Beijing: Chemical Industry Press, pp. 133−134.

Ashraf M, Foolad MR. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, **59**: 206−216.

Barceló J, Poschenrieder C. 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminum toxicity and resistance: a review. *Environmental and Experimental Botany*, **48**: 75−92.

Basu U, Good AG, Taylor GJ. 2001. Transgenic *Brassica napus* plants overexpressing aluminum-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminum. *Plant Cell Environment*, **24**: 1269−1278.

Chen HJ, Li YQ, Chen DD, Zhang Y, Wu LM, Ji JS. 1996. Soil phosphorus fractions and their availability in Chinese fir plantations in south China. *Forestry Research*, **9**: 121−126.

Foyer CH, Noctor G. 2005. Oxidant and antioxidant signaling in plants: a reevaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environment*, **28**: 1056−1071.

Gao Y, Wu X, Sun M. 2009. Effects of ectomycorrhizal seedlings of masson pine on absorption and utilization of N, P and K. *Journal of Nanjing Forestry University*, **33**(4): 33−36.

Gaume A, Mächler F, De Léon C, Narro L, Frossard E. 2001. Low-P tolerance by maize (*Zea mays* L.) genotypes: Significance of root growth, and organic acids and acid phosphatase root exudation. *Plant and Soil*, **228**: 253−264.

Giannakoula A, Moustakas M, Syrosb T, Yupsanisb T. 2011. Aluminum stress induces up-regulation of an efficient antioxidant system in the Al-tolerant maize line but not in the Al-sensitive line. *Environmental and Experimental Botany*, **67**: 487−494.

Kochian LV, Hoekenga OA, Piñeros MA. 2004. How do crop plants tolerate acid soils? —Mechanisms of aluminum tolerance and phosphorous efficiency. *Annual Review of Plant Biology*, **55**: 459−493.

Li SF, Liu ST, Zhou JP. 2007. Measurement of catalase vigor in plants with spectrophotometry. *Anhui Agricultural Science Bulletin*, **13** (2): 72−73.

Mao DR. 2005. Plant nutrition research methods. Beijing: China Agricultural University Press, pp. 20−55.

Mclachlan KD. 1980. Acid phosphatase activity of intact roots and phosphorus nutrition in plants (I): Assay conditions and phosphatase activity. *Australian Journal of Agricultural Research*, **31**: 42−440. ()

Ogawa T, Matsumoto C, Takenaka C, Tezuka T. 2000. Effect of Ca on Al-induced activation of antioxidant enzymes in the needles of hinoki cypress (*Chamaecyparis obtusa*). *Journal of Forest Research*, **5**: 81−85.

Poschenrieder C, Gunsé B, Corrales I, Barceló J. 2008. A glance into aluminum toxicity and resistance in plants. *Science of the total environment*, **400**: 356−368.

Raghothama KG, Karthikeyan AS. 2005. Phosphate acquisition. *Plant and Soil*, **274**: 37-49.

Raghothama KG. 1999. Phosphate acquisition. *Annual Review of Plant Physiology and Plant Molecular Biology*, **50**(1): 665−693.

Schachtman DP, Reid RJ, Ayling SM. 1998. Phosphorus uptake by plants: from soil to cell. *Plant Physiology*, **116**: 447−453.

Sharma SS, Dietz KJ. 2006. The significance of amino acids and amino acid derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany*, **57**: 711−726.

Shi J, Gu Y, Zhao, Q. 2006. Research on the mycorrhiza fungi of *Pinus massoniana* Lamb. Evergreen broad-leaved forests in Tainting, Zhejiang province. *Journal of Central China University*, **26**(1): 41−44.

Singh D, Sale PWG, Pallaghy CK, Singh V. 2000. Role of proline and leaf expansion rate in the recovery of stressed white clover leaves with increased phosphorus concentration. *New Phytologist*, **146**: 26−269.

Smith FW, Jackson WA, Vanden Berg PJ. 1990. Internal phosphorus flows during development of phosphorus stress in *Stylosanthes hamata*. *Australian Journal of Plant Physiology*, **17**: 451−464.

Snapp S, Lynch JP. 1996. Phosphorus distribution and remobilization in bean plants as influenced by phosphorus nutrition. *Crop Science*, **36**: 929−935.

Vance CP, Uhde-Stone C, Allan DL. 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytologist*, **157**: 423−447.

Veljovic-Jovanovic S, Kukavica B, Stevanovic B, Navari-Izzo F. 2006. Senescence and drought-related changes in peroxidase and superoxide dismutase isoforms in leaves of *Ramonda serbica*. *Journal of Experimental Botany*, **57**: 1759−1768.

Vincent JB, Crowder MW, Averill BA. 1992. Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *Trends in Biochemical Science*, **17**: 105−110.

Wang J, Han XR, Zhan XM, Hou YH, Zhao WL. 2005. Effect of low-phosphorus stress on membrane lipid peroxidation and the protection of enzyme activities in tomato leaves. *Plant Nutrition and Fertilizer Science*, **11**(6): 851−854.

Wang Y, Ding G. 2011. Effects of exogenous mycorrhiza on growth of Pinus massoniana seedlings. *Journal of Central South University of Forestry and Technology*, **31**(4): 31−34.

Xie YR, Jin GQ, Chen Y, Song ZY. 2005. Study on phosphorus efficiency of different provenances of *Pinus massoniana*. *Scientia Silver Sinicae*, **41**(4): 25−30.

Xie YR, Zhou ZC. 2002. Research advance on adaptation mechanism of forest tree to low-phosphorus stress and genetics of phosphorus efficiency. *Forest Research*, **15**(6):734−740.

Xu XH, Ding GJ. 2006. Physiological and biochemical responses of *Pinus massoniana* to low phosphorus stress. *Scientia Silver Sinicae*, **42**(9): 24−28.

Yan X, Liao H, Trull MC, Beebe SE, Lynch JP. 2001. Induction of a major leaf acid phosphatase does not confer adaptation to low phosphorus availability in common bean. *Plant Physiology*, **125**: 1901−1911.

Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K. 1997. Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell and Physiology*, **38**: 1095−1102.

Yu YC, Yu J, Fang L, Shan Q, Jiang DF. 2007. Organic acids exudation from the roots of *Cunninghamia lanceolata* and *Pinus massoniana* seedlings under low phosphorus stress. *Journal of Nanjing Forestry University*, **31**(2): 9−12.

Zhang ZX. 1986. Determination of chlorophyll content of plants - acetone and ethanol mixture method. *Liaoning Agricultural Science*, **3**: 26−28.

Zheng BS. 2006. Plant physiology and biochemistry of modern research techniques. *Meteorological Press*, 107−109.

Zhou ZC, Jin GQ, Chen, Y. 2003. Kinetics of phosphorus uptake by different provenances of Masson Pine under low phosphorus stress. *Forest Research,* **16**(5): 548−553.

Zhou ZC, Liao GH, Jin GQ, Chen Y. 2005. Difference of induced acid phosphate activity under low phosphorus stress of *Pinus massoniana* provenances. *Scientia Silver Sinicae*, **41**(3): 58−62.

Zhou ZC, Xie YR, Jin GQ, Wang YS, Hong GM. 2004. Inheritance and variation of phosphorus efficiency and its related traits in families of *Pinus massoniana*. *Journal of Beijing Forestry University*, **26**(6): 1−5